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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket Number 059440/0128

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In re patent application of
Brian S. Hooker *et al.*

Serial No.: 09/588,314

Filed: June 6, 2000

Group Art Unit: 1635

Examiner: M. Schmidt

For: METHOD OF PRODUCING HUMAN COAGULATION FACTOR FROM WHOLE PLANTS OR PLANT CELL CULTURES

DECLARATION UNDER 37 CFR §1.132 OF DR. Brian S Hooker

I, Brian S. Hooker of 503 S. Young Place, Kennewick, WA U.S.A., declare that:

1. I received my Ph.D in 1990 from Washington State University, Doctor of Biochemical Engineering.

2. I am employed, as a director of research for a biotechnology company.

3. I have been doing research and development in the field of Plant Molecular Farming.

4. My curriculum vitae and a list of my publications are appended hereto as APPENDIX A.

5. I am a co-inventor of U.S. Application Serial Number 09/588,314 ("the application"). In relation to the application, I have read the Office Action mailed October 2, 2001.

6. The instant application claims a method of producing a human coagulation factor VIII in a transgenic plant. Human factor VIII is the largest single-frame protein expressed to date in a transgenic plant. Each subunit of the largest multiple subunit protein to be expressed *in planta* to date, is not larger than 75 kDa. See Frigerio et al, Plant Physiol. 2000 Aug;123(4):1483-94.

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In contrast, the human factor VIII protein is greater than 300 kDa when correctly processed, each individual heavy chain is over 220 kDa as indicated by the Western blot immunoassay. The individual heavy chain shown as derived from tobacco plant leaf samples is depicted in a Western blot assay on Figure 4 in the specification.

7. As described in Example 1 of the specification, my co-workers and I used *Agrobacterium*-mediated transformation to make a tobacco plant transformed with vector pZD201. The expression of human factor VIII in mature tobacco plants and calli was tested. A dot blot assay, using leaf protein extracts showed the presence of factor VIII antigen in the leaf tissues of T0 whole plant transformants, plants 1004-3, 1006-2 and 1006-3. See Figure 3 of the specification. We self-pollinated T0 plants to obtain T1 seedstock. Protein extracts from leaves of T1 plants selected for kanamycin resistance were assayed by Western immunoblotting. The results are shown in Figure 4 of the specification. We observed comparable immunoreactive bands between human factor VIII produced in plant and from plasma. Therefore, the plant-derived human factor VIII undergoes correct post-translational modification resulting in a bioactive human factor VIII.

8. My co-workers and I conducted an assay to confirm the bioactivity of the human factor VIII produced in the tobacco plants described in paragraph 7. We harvested the transgenic plant leaf material and extracted total soluble protein using standard techniques. Functionality of the recombinant human factor VIII was analyzed using the Coatest method (Helena Laboratories, Beaumont, Texas). After activation by thrombin, factor VIII acts as a cofactor in the conversion of factor X to factor Xa by factor IXa when calcium and phospholipid are present. In the Coatest assay, the quantity of factor Xa generated was determined using a specific chromogenic substrate (MeO-CO-D-CHG-Gly-Arg-pNa) and was directly proportional to the amount of factor VIII in the sample tested. During the functional analysis, total protein samples from plants expressing factor VIII protein, as well as untransformed control plants and CBHI cellulase expressing tobacco plants, were tested.

9. Results of the Coatest assay are shown in the following table. In each individual sample, 1.5 mg of soluble plant protein was used. Tests A, B and C were completed on separate days and each required a separate untransformed plant control. In tests A and B, the duration of incubation after factor Xa addition was 5 minutes. In test C, the duration of incubation after factor Xa addition was 4 minutes. In addition, in test C,

aliquots of factor VIII reference plasma standard (Helena Laboratories, Beaumont, TX) were added to two separate tobacco plant controls. Results from tests A and B, as compared to increases in absorbance mediated by the addition of factor VIII in test C, clearly show the presence of factor VIII procoagulant activity in tobacco plant lines 1005-5, 1005-6, and 1006-3. The highest level of activity observed in line 1006-3 would roughly correspond to 26 ng of factor VIII per 1500 mg sample (based on linear regression of calibration data from test C) or an expression level of 0.002% of extractable leaf protein. Therefore, the results of this test further demonstrate that the recombinant human factor VIII produced by the claimed method is correctly processed *in planta*, to produce a protein with procoagulant activity.

Results of Coatest Assay for selected plant transformants

Test	Plant Line	Change in Absorbance Upon addition of Factor X_a (A_{405})	Change in Absorbance Compared to Plant Control (A_{405}[sample] - A_{405}[control])
A	1005-5	0.322	0.118
A	1005-6	0.269	0.065
A	plant control	0.204	-
B	1006-3	0.676	0.239
B	plant control	0.437	-
C	plant control 1 w/ 5 ng FVIII	0.134	0.106
C	plant control 1 w/ 10 ng FVIII	0.176	0.148
C	plant control 1 w/ 30 ng FVIII	0.268	0.24
C	plant control 1	0.028	-
C	plant control 2 w/ 5 ng FVIII	0.177	0.137
C	plant control 2 w/ 10 ng FVIII	0.222	0.182
C	plant control 2 w/ 30 ng FVIII	0.305	0.265
C	plant control 2	0.040	-

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

4/1/02
Date

B.S. Hooker
Dr. Brian S. Hooker

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EDUCATION

Ph.D., Washington State University (Pullman, WA), Biochemical Engineering, 1990
M.S., Washington State University (Pullman, WA), Biochemical Engineering, 1988
B.S., California State Polytechnic University (Pomona, CA), Chemical Engineering, 1985

PROFESSIONAL LICENSE

Licensed Professional Engineer, Chemical Engineering, State of Washington

EXPERIENCE

PhytaGenics, Inc. – July 2000 to present, (Director of Research) Responsible for research and development programs focused on recombinant pharmaceutical protein production via transgenic plants as well as over pharmaceutical product development. Other responsibilities include oversight of gene discovery in plants and metabolic engineering for the production of important phytochemicals.

Battelle/Pacific Northwest National Laboratory (Richland, WA), Environmental Technology Division - November 1993 to January 2001, Responsible for applied plant and yeast molecular biology programs including development of transgenic production systems for human pharmaceutical proteins and industrial enzymes. In addition, am responsible for deployment technologies for enzyme delivery systems in industrial, chemical and medical applications. Formerly responsible for microbial kinetic and transport modeling, design, development and support for the demonstration of in situ/ex situ biological destruction of chlorinated organic hydrocarbons.

Tri-State University (Angola, IN), Department of Chemical Engineering, Assistant Professor - From September 1990 to November 1993, Responsible for formulation and teaching of undergraduate chemical engineering coursework as well as the development of a full funded biotechnology/bioremediation laboratory to provide educational and research experiences for engineering and science students.

Battelle, Pacific Northwest Laboratory (Richland, WA), NORCUS, Faculty Fellow - From Summer 1992, 1993 (Full-time), Fall 1992, Spring 1993, Fall 1993 (Part-Time), Led an investigation of the kinetics and transport phenomena associated with in situ microbial chlorinated hydrocarbons degradation under anaerobic conditions. Formulated a mathematical model of carbon tetrachloride destruction in fed-batch systems.

Washington State University (Pullman, WA), Department of Chemical Engineering, Teaching Assistant - Fall 1989, Taught undergraduate Biochemical Engineering, a senior level technical elective. Fall 1986-Spring 1987, Assisted students in operating equipment and in general experimental procedures within Unit Operations laboratory.

INDUSTRIAL CONSULTING

BioRem, Inc. (1991-1993), Consulted on a variety of studies associated with commercial scale in situ bioremediation, including toxicity and treatability testing.

Voltek, Div. of Sekisui Chemical Co. (Fall 1991), Consulted on the retrofit of catalyst beds within an emissions control incinerator on a plastics foaming oven.

RESEARCH GRANTS AND CONTRACTS

"Production of plant-based recombinant thrombin," National Institutes of Health Phase I SBIR Grant, 2002.

"Production of plant-based IgG monoclonal antibody," Contract with Epicyte Pharmaceuticals, 2001-2004

"Production of plant-based elastin biomaterials," U.S. Army Institute of Surgical Research, 2001-2003

"Conversion of Starch Waste to 1,3 Propanediol," Joint Funding Project by the Environmental Technology Initiative and the Energy Technology Initiative, Pacific Northwest National Laboratory, 1998.

"Discovery, Isolation and Characterization of Strong Plant Promoters," Food and Agriculture Initiative, Pacific Northwest National Laboratory, 1997-2000.

"Engineered Biomaterials for Accelerated Wound Care," Medical Technologies and Systems Initiative, Pacific Northwest National Laboratory, 1997-1998

"Enzyme Technologies," Biomolecular Technologies Initiative, Core Technical Investment, Battelle Memorial Institute, 1997-1999.

"Production of Human Blood Coagulation Factors in Transgenic Plants," Battelle Independent Research and Development, 1997-2000.

"Production of Cellulases in Transgenic Crop Plants, DOE-EE, Biofuels Program, NREL Ethanol Project, 1997-1999.

"Genetically Engineered Sensors, Level V Scientist LDRD, Pacific Northwest National Laboratory, 1996.

"Production of Foreign Protein Pharmaceuticals from Transgenic Plant Cell Culture," Environmental Technology Division, Pacific Northwest National Laboratory, 1996.

"Remediation Technology Development Forum," Department of Energy, Office of Technology Development, Plumes Focus Area, 1996-1998.

"VOC Arid/Integrated Demonstration of Carbon Tetrachloride and Nitrate Destruction," Department of Energy, Office of Technology Development, 1993-1996.

"Mixed Waste Treatment Using Iron Reducing Bacteria," Microbial Biotechnology Initiative, Pacific Northwest National Laboratory 1996-1997.

"Characterization of Efficient Dechlorinators: Destruction of NAPL," Microbial Biotechnology Initiative, Pacific Northwest National Laboratory, 1995-1997.

"Formulation and Evaluation of Simusolv Chemical Engineering Applications Guide," Dow Chemical Company, 1993-1995.

"Techniques for remediation of Industrial Waste Substances II," Lilly Faculty Development Grant, 1993.

"Application of Structured Kinetic Modeling to the Microbial Destruction of CCl_4 in Hanford Groundwater," Battelle Pacific Northwest Laboratory, 1992-1993.

"Plant Tissue Cultivation and In Situ Bioremediation Equipment," Olive B. Cole Foundation, 1992-1993.

"Techniques for Remediation of Industrial Waste Substances," Lilly Faculty Development Grant, 1992-1993.

"Development and Implementation of a Biochemical Engineering Laboratory at Tri-State University," National Science Foundation, 1991-1994.

"Development of an Experiment in Enzymatic Cellulose Hydrolysis," Tri-State University Faculty Small Grants Program, 1991.

AWARDS/HONORS

- Entrepeneurial Award, Battelle, 2001
- Invited Presentation, "Phytobiotechnology: Prospects and Promise," Western Association of Food and Drug Officials Annual Meeting, 2001
- Federal Laboratory Consortium Recognition Award, "Reactive Transport in 3-Dimensions," 1999
- Outstanding Performance Award, Pacific Northwest National Laboratory "Reactive Transport in 3-Dimensions," 1999
- Outstanding Performance Award, Pacific Northwest National Laboratory, "Demonstration of In Situ Bioremediation of Carbon Tetrachloride," 1997
- Invited Presentation, "Technology and IP Development in an Integrated Business Model," Battelle Strategic Leadership Conference, November, 1997
- Recipient, McKetta-Smith Teaching Excellence Award, 1993

PATENTS

1. Dai Z, Shi L, Hooker BS. Epimerase Gene and Use Thereof. Filed January 2001.

2. Dai Z, Shi L, Hooker BS. Gene Promoters Isolated from Potato and Use Thereof. Filed January 2001.

3. Hooker BS, Anderson DB, Gao J, Dai, Z. Controlled Environment Agriculture Bioreactor For Heterologous Protein Production. Filed July 2000.

4. Shi L, Hooker BS, Dai Z, Anderson, DB, Gao J. Tobacco Ribosomal Protein L-25 Promoter, Pending, Filed December 1999.

5. Shi L, Dai Z, Gao J, Hooker BS, Anderson DB. US Patent **6,355,864** Versatile rpL34 elements and use thereof. Issued: March 12, 2002; filed October 12, 1999.

6. Gao J, Skeen RS, Hooker BS, Anderson, DB. Genetically Engineered Microorganisms Useful in Expression of Bioproducts from Starch Waste. Pending, Filed February 1999.

7. Hooker BS, Gao J, Anderson, DB, Dai Z. Transgenic Plant-Derived Human Blood Coagulation Factors. ALLOWED, Filed May, 1998, CIP filed May 1999.

TECHNICAL PUBLICATIONS

1. Dai Z, Hooker BS, Quesenberry RD, Thomas SR. 2001. Expression of *Acidothermus cellulolyticus* endoglucanase (E1) in transgenic tobacco plants is enhanced by post-transcriptional modification. Submitted for publication.

2. Dai Z, Hooker BS, Anderson, DB, Thomas SR. 2000. Improved plant-based production of E1 endoglucanase using potato:expression optimization and tissue targeting. Mol Breeding 6:277-285.

3. Hooker BS, Dai Z, Anderson DB, Quesenberry RD, Ruth MF, Thomas SR. 2000. Production of Microbial Cellulases in Transgenic Crop Plants. In: Glycosyl Hydrolases for Biomass Conversion. Himmel ME, Baker JO, Saddler JN, Eds. ACS Symposium Series 769:55-90.

4. Dai Z, Hooker BS, Anderson DB, Thomas SR. 2000. Expression of *Acidothermus cellulolyticus* endoglucanase (E1) and its biochemical characteristics in transgenic tobacco. Transgenic Res. 9:43-54.

5. Gao J, Hooker BS, Skeen RS, Anderson DB. 1999. Transgenic fungal-based conversion of waste starch to industrial enzymes. Proceedings of the 4th Biomass Conference of the Americas, pp. 895-901.

6. Hooker BS, Skeen RS. 1999. Transgenic phytoremediation blasts onto the scene. Nat Biotechnol. 17:428.

7. Dai Z, Hooker BS, Quesenberry RD, Gao J. 1999. Expression of *Trichoderma reesei* exo-cellobiohydrolase I (CBH I) in transgenic tobacco leaves and calli. Appl Biochem Biotechnol

77-79:689-699.

8. Sun Y, Petersen JN, Bear J, Clement TP, Hooker BS. 1999. Modeling microbial transport and biodegradation in a dual-porosity system, TIPM 35(1):49-65.

9. Hooker BS, Skeen RS, Truex MJ, Johnson CD, Peyton BM, Anderson DB. 1998. In situ bioremediation of carbon tetrachloride: field test results. Bioremediation. 1:181-193.

10. Clement TP, Sun Y, Hooker BS, Petersen JN. 1998. Modeling multi-species reactive transport in groundwater aquifers, Groundwater Monitoring and Remediation Journal 18:79-92.

11. Sun Y, Petersen JN, Clement TP, Hooker BS. 1998. Effect of reaction kinetics on predicted concentration profiles during subsurface bioremediation, J Contam Hydrol 31:147-162.

12. Franzen MEL, Petersen JN, Clement TP, Hooker BS, Skeen RS. 1997. Pulsing of multiple nutrients as a strategy to achieve large biologically active zones during in situ carbon tetrachloride remediation. Computational Geosciences, 1:271-288.

13. Gao J, Skeen RS, Hooker BS, Quesenberry RD. 1997. Effects of several electron donors on tetrachloroethylene dechlorination in anaerobic soil microcosms. Water Res 31:2479-2486.

14. Clement, T. P., Y. Sun, B. S. Hooker, and J. N. Petersen. 1997. Modeling Natural Attenuation of Contaminants in Saturated Groundwater. *Proc. 4rd International Symposium on In Situ and On Site Bioremediation.* **1**:37-42.

15. Clement, T. P., M. J. Truex, and B. S. Hooker. 1997. A Two-Well, Steady-State Testing Method for Determining Hydraulic Properties of Confined and Unconfined Aquifers. *Ground Water* **35**:698-703.

16. Clement, T. P., B. S. Hooker, and R. S. Skeen. 1996. Macroscopic Models for Predicting Changes in Saturated Porous Media Properties Caused by Microbial Growth. *Ground Water* **34**:934-942.

17. Clement, T. P., B. S. Hooker, and R. S. Skeen. 1996. Numerical Simulation of Biologically Reactive Transport From a Nutrient Injection Well. *ASCE J. Env. Eng.* **122**:833-839.

18. Hooker, B. S. and R. S. Skeen. 1996. Intrinsic Remediation: An Environmental Restoration Technology. *Curr. Opin. Biotechnol.* **7**:317-320.

19. Skeen, R. S., J. Gao, and B. S. Hooker. 1995. Kinetics of Chlorinated Ethylene Dehalogenation under Methanogenic Conditions. *Biotechnol. Bioeng.* **48**:659-666.

20. Gao, J., R. S. Skeen, B. S. Hooker. 1995. Effects of Temperature on Perchloroethylene Dechlorination by a Methanogenic Consortium. *Proc. 3rd International Symposium on In Situ and On Site Bioreclamation.* **4**:53-59.

21. Jennings, D. A., J. N. Petersen, R. S. Skeen, B. M. Peyton, B. S. Hooker, T. P. Clement, D. L. Johnstone, and D. R. Yonge. 1995. An Experimental Study of Microbial Transport in Porous Media. *Proc. 3rd International Symposium on In Situ and On Site Bioreclamation.* **3**:97-103.

22. Sherwood, J. L., J. N. Petersen, R. S. Skeen, and B. S. Hooker. 1995. Effect of Nitrate Availability on Chloroform Production During Carbon Tetrachloride Destruction. *Proc. 3rd International Symposium on In Situ and On Site Bioreclamation.* **4**:85-89.

23. Peyton, B. M., M. J. Truex, R. S. Skeen, and B. S. Hooker. 1995. The Use of Bench and Field-Scale Data for the Design of an In Situ Carbon Tetrachloride Bioremediation System. *Proc. 3rd International Symposium on In Situ and On Site Bioreclamation.* **4**:111-116.

24. Clement, T. P., B. S. Hooker, and R. S. Skeen. 1995. Modeling Biologically Reactive Transport in Porous Media. *Proceedings of the International Conference on Mathematics and Computations, Reactor Physics, and Environmental Analyses*, Portland, Oregon. 192-201.

25. Peyton, B. M., R. S. Skeen, B. S. Hooker, R. W. Lundman, and A. B. Cunningham. 1995. Evaluation of Bacterial Detachment Rates In Porous Media. *Applied Biochem. Biotechnol.* **51**:785-797.

26. Jennings, D. A., J. N. Petersen, R. S. Skeen, B. S. Hooker, B. M. Peyton, D. J. Johnstone, and D. R. Yonge. 1995. Effects of Slight Variations in Nutrient Loadings on Pore Plugging in Soil Columns. *Applied Biochem. Biotechnol.* **51**:727-734.

27. Skeen, R. S., N. B. Valentine, B. S. Hooker, and J. N. Petersen. 1995. Kinetics of Nitrate Inhibition of Biological Transformation of Carbon Tetrachloride. *Biotechnol. Bioeng.* **45**:279-284.

28. Hooker, B.S., R.S. Skeen, M.J. Truex, and B.M. Peyton. 1994. A Demonstration of *In Situ* Bioremediation of CCl_4 at the Hanford Site. pp. 281-292 in: *In Situ Remediation: Scientific Basis for Current and Future Technologies, Part 1*, G. W. Gee and N. R. Wing, eds., Battelle Press, Columbus.

29. Truex, M. J., C. D. Johnson, D. R. Newcomer, L. A. Doremus, B. S. Hooker, B. M. Peyton, R. S. Skeen, and A. Chilikapati. 1994. Deploying *In Situ* Bioremediation at the Hanford Site. pp. 209-231 in: *In Situ Remediation: Scientific Basis for Current and Future Technologies, Part 1*, G. W. Gee and N. R. Wing, eds., Battelle Press, Columbus.

30. Shouche, M. A., J. N. Petersen, R. S. Skeen and B. S. Hooker. 1994. Alternating Extraction/Injection Well Interactions for In Situ Bioremediation. *Appl. Micro. Biotechnol.* **45**:775-85.

31. Hooker, B. S., R. S. Skeen and J. N. Petersen. 1994. Biological Destruction of CCl_4, Part II: Kinetic Modeling. *Biotechnol. Bioeng.* **44**:211-218.

32. Hooker, B. S. 1994. A Project Oriented Approach to an Undergraduate Biochemical Engineering Laboratory. *Chem. Eng. Educ.* **28**:98-102.

33. Petersen, J. N., R. S. Skeen, K. M. Amos, and B. S. Hooker. 1994. Biological Destruction of CCl_4: Part 1. Experimental Design and Data. *Biotechnol. Bioeng.* **43**:521-528.

34. Hooker, B. S., R. S. Skeen, and J. N. Petersen. 1994. Application of a Structured Kinetic Model to the Bioremediation of Hanford Groundwater, pp. 387-392. In: *Bioremediation for Chlorinated and*

Polycyclic Aromatic Hydrocarbon Compounds. R.E. Hinchee *et al.*, eds. Lewis, Boca Raton, FL.

35. Skeen, R. S., S. P. Luttrell, T. M. Brouns, B. S. Hooker, M. Shouche, and J. N. Petersen. 1993. In Situ Bioremediation of Hanford Groundwater. *Remediation*, **3**:353.367.

36. Hooker, B. S. and J. M. Lee. 1992. Application of a New Structured Model to Tobacco Cell Cultures. *Biotechnol. Bioeng.*, **39**:765-774.

37. Hooker, B.S., J. M. Lee and G. An. 1990. Cultivation of Plant Cells in a Stirred Vessel: Effect of Impeller Design. *Biotechnol. Bioeng.*, **35**:296-304.

38. Hooker, B. S. and J. M. Lee. 1990. Cultivation of Plant Cells in Aqueous Two-Phase Polymer Systems. *Plant Cell Reports*, **8**:546-549.

39. Hooker, B. S., J. M. Lee and G. An. 1989. The Response of Plant Tissue Culture to a High Shear Environment. *Enzy. Microb. Technol.*, **11**:484-490.

PRESENTATIONS

1. Hooker BS. Phytobiotechnology: Prospects and Promise. **Invited Presentation** at the 2000 Western Association of Food and Drug Officials Annual Meeting, Seattle, WA, September, 2000.
2. Hooker BS, Anderson DB, Gao J. Plant bioreactors for recombinant human blood factors production. Presented at the Annual Biotechnology Industry Organization Meeting, Boston, MA, March, 2000.
3. Hooker BS, Anderson DB, Dai Z. Plant promoter technology program at Battelle. Presented at the Annual Biotechnology Industry Organization Meeting, Boston, MA, March, 2000.
4. Shi L, Hooker BS, Quesenberry RD, An G, Dai Z. Functional analysis of promoter elements controlling developmental and environmental regulation of a tobacco ribosomal protein gene *L34*. Presented at the 1999 American Society of Plant Physiologists Annual Meeting, Baltimore, MD, July, 1999.
5. Dai Z, Hooker BS, Thomas SR. Expression of Acidothermus cellulolyticus endoglucanase (E1) and its biochemical characteristics in transgenic tobacco. Presented at the 1999 American Society of Plant Physiologists Annual Meeting, Baltimore, MD, July, 1999.
6. Hooker BS, Gao J, Dai Z, Anderson DB, Quesenberry RD. Plant-based recombinant human blood factors. Presented at the International Business Communications AgBiotech World Forum, Las Vegas, NV, June, 1999
7. Hooker BS, Anderson, DB. Plant bioreactors for recombinant protein production: technical and economical analysis. Presented at the Annual Biotechnology Industry Organization Meeting, Seattle, WA, May 1999.
8. Dai Z, Hooker BS, Quesenberry RD, Thomas SR. Expression of *Acidothermus cellulolyticus* endoglucanase (E1) in transgenic tobacco plants is enhanced by post-transcriptional modification. Presented at the 21th Symposium on Biotechnology of Fuels and Chemicals, Fort Collins, CO, May,

1999.

9. Dai Z, Hooker BS, Quesenberry RD, Thomas SR. Expression of *Acidothermus cellulolyticus* endoglucanase (E1) and its biochemical characteristics in transgenic tobacco. Presented at the 21th Symposium on Biotechnology of Fuels and Chemicals, Fort Collins, CO, May, 1999.

10. Ruth MF, Howard JA, Nikolov ZL, Hooker BS, Himmel ME, Thomas SR. Preliminary economic analysis of agricultural enzyme production for use in cellulose hydrolysis. Presented at the 21th Symposium on Biotechnology of Fuels and Chemicals, Fort Collins, CO, May, 1999.

11. Gao J, Skeen RS, Hooker BS, Anderson DB. Transgenic fungal-based conversion of waste starch to industrial enzymes. Presented at the 21th Symposium on Biotechnology of Fuels and Chemicals, Fort Collins, CO, May, 1999.

12. Hooker BS, Z Dai, RD Quesenberry, SR Thomas. Production of Cellulases in Transgenic Tobacco Whole Plants: Production Optimization at the Molecular Level. Presented at the 20th Symposium on Biotechnology of Fuels and Chemicals, Gatlinburg, TN, May, 1998.

13. Dai, Z., J. Gao, B. S. Hooker. Expression of *Trichoderma reesei* exocellobiohydrolase I in transgenic tobacco plants. Presented at the Quadrennial Joint Annual Meetings of The American Society of Plant Physiologists and Canadian Society of Plant Physiologists with the participation of the Japanese Society of Plant Physiologists and The Australian Society of Plant Physiologists, Inc, Vancouver, BC, Canada, August 1997.

14. Hooker, B. S., Z. Dai, J. Gao. Production of Cellulases in Transgenic Tobacco Whole Plants and Cell Culture. Presented at the 19th Symposium on Biotechnology of Fuels and Chemicals, Colorado Springs, CO, May, 1997.

15. Clement, T. P., Y. Sun, B. S. Hooker, and J. N. Petersen. 1997. Modeling Natural Attenuation of Contaminants in Saturated Groundwater. Presented at the 4th International Symposium on In Situ and On Site Bioremediation, New Orleans, LA, April, 1997.

16. Hooker, B. S., M. J. Truex, R. S. Skeen, B. M. Peyton, C. D. Johnson, and D. B. Anderson. 1997. Field Results for In Situ Bioremediation of Carbon Tetrachloride. Presented at the 4th International Symposium on In Situ and On Site Bioremediation, New Orleans, LA, April, 1997.

17. Gao, J., R. S. Skeen, and B. S. Hooker. 1997. Screening of Indigenous Potential for Complete In Situ Destruction of Tetrachloroethylene. Presented at the 4th International Symposium on In Situ and On Site Bioremediation, New Orleans, LA, April, 1997.

18. Jerger, D. E., D. B. Anderson, B. S. Hooker and R. S. Skeen. 1997. Scale-up of In Situ Bioremediation for Chloroethenes. Presented at the 4th International Symposium on In Situ and On Site Bioremediation, New Orleans, LA, April, 1997.

19. Gao, J., R. S. Skeen, and B. S. Hooker. 1997. Process Scale-Up Considerations for In Situ Reductive Dechlorination of Chloroethenes. Presented at the 4th International Symposium on In Situ and On Site Bioremediation, New Orleans, LA, April, 1997.

20. Peyton, B. M., B. S. Hooker, M. J. Truex, and M. G. Butcher. 1997. Pulsed Nutrient Injection for Improved Biomass Distribution. Presented at the 4th International Symposium on In Situ and On Site Bioremediation, New Orleans, LA, April, 1997.

21. Dai, Z., J. Gao, B. S. Hooker. Production and Characterization of Foreign Proteins in Transgenic Whole Plants and Cell Culture. Presented at the IBC Transgenic Therapeutics Conference, West Palm Beach, FL, February, 1997.

22. Sun, Y., T. P. Clement, B. S. Hooker, J. N. Petersen. A Modular Computer Model for Simulating Natural Attenuated of Chlorinated Organics in Saturated Groundwater. Presented at the IBC Environmental Monitoring Tools Conference, Annapolis, MD, December, 1996.

23. Dai, Z., J. Gao, B. S. Hooker. Production and Characterization of Foreign Protein Pharmaceuticals in Transgenic Whole Plants and Cell Culture. Presented at the 1996 AIChE Annual Meeting, Chicago, IL, November, 1996.

24. Sun, Y., T. P. Clement, B. S. Hooker, J. N. Petersen. A Modular Computer Model for Simulating Natural Attenuated of Chlorinated Organics in Saturated Groundwater. Presented at the Symposium on Natural Attenuation of Chlorinated Organics in Ground Water, Dallas, TX, September, 1996.

25. Hooker, B. S., M. J. Truex, R. S. Skeen, B. M. Peyton, and T. P. Clement. Comparison of Model Predictions to Field Data for a Demonstration of Carbon Tetrachloride Bioremediation. Presented at the 1996 World Congress of Chemical Engineering, San Diego, CA, July, 1996.

26. Hooker, B. S., M. J. Truex, T. P. Clement, and D. R. Newcomer. Preliminary Validation of Intrinsic Remediation of Carbon Tetrachloride at the Hanford Site. Presented at the Intrinsic Remediation Conference, Salt Lake City, UT, April 1996.

27. Hooker, B. S., M. J. Truex, B. M. Peyton, R. S. Skeen, and T. P. Clement. Validation of In Situ Bioremediation Using Numerical Simulation Tools. Presented at the 1995 AIChE Annual Meeting, Miami, FL, November, 1995.

28. Hooker, B. S., R. S. Skeen, J. Gao, M. M. Shah. Kinetic Characterization of a Tetrachloroethylene Utilizing Microbial Consortium. Presented at the 34th Hanford Symposium on Health and the Environment, Pasco, WA, October, 1995.

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PROFESSIONAL DEVELOPMENT ACTIVITIES

Business Area Leader, Transgenic Organisms, Food and Agriculture Initiative, Pacific Northwest National Laboratory, 1997.

Session Chair, "General Biotechnology Division Poster Session," AIChE Annual Meeting, Chicago, IL, November, 1996.

Session Chair, "Biological Processes Applied to Remediation and Emission Control," 5th World Congress of Chemical Engineering, San Diego, CA, July, 1996.

Session Chair, "Advances in Bioremediation of Chlorinated Organic Compounds," AIChE Annual Meeting, Miami, FL, November, 1995.

Session Co-Chair, "General Environmental Division Poster Session," AIChE Annual Meeting, Miami, FL, November, 1995.

Session Co-Chair, "Advances in Field Scale Bioremediation," AIChE Summer National Meeting, Boston, MA, August, 1995.

Short Course Attendance, "Advanced Ground Water Modeling Using MODFLOW/MT3D," International Ground Water Modeling Center, Golden, CO, June, 1995.

Invited Seminar Presentations, "Modeling of In Situ Bioremediation at Pacific Northwest Laboratory," at University of Arizona, Tucson, AZ, and Washington State University, Pullman, WA, October, 1994.

Program Chairman, New Engineering Educators Committee, American Society for Engineering Education Annual Meeting, Urbana, IL, June 1993.

Short Course Attendance, "Groundwater and Soil Remediation," AIChE Annual Meeting, Miami, FL, November 1992.

Session Co-Chair, "New Engineering Educators: Tricks of the Trade-Outside of Classroom," American Society for Engineering Education Annual Meeting, Toledo, OH, June 1992.

Short Course Attendance, "Biotechnology Plant Design," AIChE Spring National Meeting, New Orleans, LA, April 1992.

Panel Reviewer, National Science Foundation Instrumentation and Laboratory Improvement Program, January 1992.

Invited Seminar Presentations, "Engineering Aspects of Plant Tissue Cultivation" at University of Toledo, Toledo, OH, and Rose-Hulman Institute of Technology Terre Haute, IN, October, 1991.

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The Vitamin K–Dependent Carboxylase[1]

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ABSTRACT The carboxylase is an integral membrane glycoprotein that uses vitamin K to modify clusters of glutamyl residues (glu's) to γ-carboxylated glutamyl residues (gla's) post-translationally in vitamin K–dependent (VKD) proteins as they pass through the endoplasmic reticulum. Carboxylation is required for VKD protein functions in hemostasis, bone metabolism, growth control and signal transduction. Carboxylation of multiple glu residues is accomplished via a processive mechanism, which occurs with at least some order and involves carboxylation of the carboxylase. The carboxylase has a high affinity binding site for VKD proteins, which in most cases is a VKD propeptide sequence; it also appears to have a low affinity site for those glu's undergoing catalysis. The propeptide activates binding of the glu's; together, the two contact points between the carboxylase and VKD protein increase the affinity of the carboxylase for vitamin K. Biochemical mapping to identify where these events occur in the carboxylase remains a challenge, despite the availability of recombinant protein. The affinity of the carboxylase for the propeptide of several VKD proteins that are coexpressed in liver varies over a 100-fold range. Treatment with anticoagulants such as warfarin that indirectly block carboxylation likely decreases the rate of VKD protein catalysis and increases the accumulation of VKD precursors, leading to a competitive state among these proteins, which results in the premature dissociation of undercarboxylated, inactive protein. J. Nutr. 130: 1877–1880, 2000.

KEY WORDS: • vitamin K • vitamin K–dependent carboxylase

The only recognized function for vitamin K (vit K)[2] in higher organisms is as a cofactor for an enzymatic reaction that converts glutamyl residues (glu's) to γ-carboxylated glutamyl residues (gla's) in a class of proteins referred to as vitamin K–dependent (VKD) proteins. These proteins are modified by the VKD- or γ-carboxylase as they are secreted through the endoplasmic reticulum (ER). A model has been proposed in which a weak base (cys) generates a strong base (e.g., vit K alkoxide) that is sufficient for the stereospecific abstraction of a hydrogen ion from the γ-glutamyl position (1). Subsequent addition of CO_2 to the carbanion intermediate produces the gla residue. Each cycle of glu to gla conversion results in the oxidation of vit K hydroquinone (KH_2) to vit K epoxide, and the carboxylase is also an epoxidase. Carboxylation, which is required for the biological activity of VKD proteins, occurs at multiple residues [3–12] within a region called the gla domain. Carboxylation results in Ca^{++}-binding and a conformational change in the gla domain, with consequent insertion of hydrophobic residues within this domain into phospholipid bilayers in which VKD proteins exert their biological effects (2).

The VKD proteins presently comprise a family of ~12 proteins. The first such proteins identified were ones involved in hemostasis, including prothrombin (PT), factor IX (fIX), factor VII (fVII), factor X (fX), protein C (PC) and protein S (PS). Subsequent identification of other VKD proteins has shown that carboxylation is important to a broader range of biological functions, including bone morphogenesis [bone gla protein (BGP) and matrix gla protein (MGP)] and growth control [(gas)6] (3,4). VKD proteins with potential functions in signal transduction [proline-rich gla protein (PRGP)-1 and PRGP-2] (5) and a VKD protein of unknown function (protein Z) have also been discovered. Finally, the carboxylase itself has been shown to be a VKD protein (6). This observation was a surprise because the carboxylase does not share any homology with the other VKD proteins. It is very likely that other VKD proteins remain to be discovered. Tissue distribution of the carboxylase is widespread, thus accommodating such a possibility.

In vivo carboxylation of VKD proteins requires the continual recycling of vit K epoxide to KH_2. Epoxide reduction is accomplished by a reductase that has been proposed to exist as a multisubunit complex with the carboxylase (7). This enzyme is inhibited by 4-OH coumarin analogs (e.g., warfarin), leading to the rapid depletion of the KH_2 supply. This extinction results in the accumulation of intracellular VKD precursors and, depending on the organism and the dose and duration of warfarin, to the secretion of undercarboxylated VKD proteins in vivo. Because of the requirement of several of the VKD proteins for normal hemostasis, these coumarins form the basis for anticoagulant therapy. Such therapy was developed initially in the absence of knowledge about VKD proteins required for biological processes other than hemostasis. Thus, long-term coumarin therapy has broader physiologic consequences than originally envisioned.

VKD Protein/Carboxylase Interaction. All VKD proteins have in common a homologous ~18 amino acid sequence; in most cases, this sequence is a propeptide juxtaposed immediately upstream of the gla domain, which targets the VKD protein to the carboxylase (8). Three of the propeptide residues (phe at −16, ala at −10 and leu at −6) are highly conserved, and mutations at these positions decrease carboxylase affinity for the VKD proteins. However, other residues are clearly important to binding. In a recent study that included the analysis of six hemostatic VKD propeptides, all possessing the three invariant residues, a 100-fold range in carboxylase affinities was observed (9).

In addition to its docking function, the propeptide is also an allosteric activator (10). Thus, incubation of a free propeptide sequence with a peptide derived from the gla domain (**Fig. 1A**) effects an increase in affinity for the gla domain peptide, but not for any of the cofactors (KH_2, O_2, CO_2). Covalent attachment of the two sequences causes a dramatic decrease in K_m for the gla domain, from the millimolar range into the low- or submicromolar

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[2] Abbreviations used: BGP, bone gla protein; ER, endoplasmic reticulum; fIX, factor IX; fVII, factor VII; fX, factor X; gas, growth arrest specific; gla, γ-carboxylated glutamyl residues; glu, glutamyl residues; KH_2, vitamin K hydroquinone; MGP, matrix gla protein; PC, protein C; PRGP, proline-rich gla protein; PS, protein S; PT, prothrombin; PZ, protein Z; r-, recombinant-; vit K, vitamin K; VKD, vitamin K dependent.

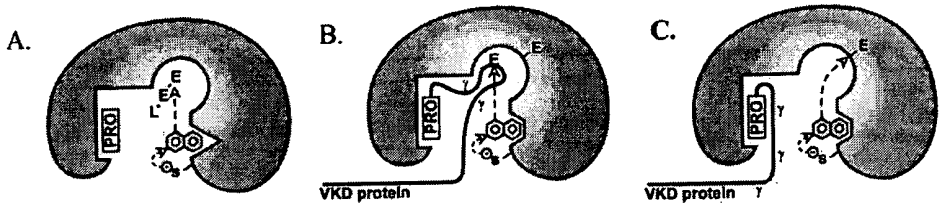


FIGURE 1 The carboxylase active site. Most vitamin K–dependent (VKD) proteins bind to the carboxylase through the propeptide (PRO), and there may also be a second point of contact for the glutamyl (glu; E) residues, as illustrated by the pocket. Covalent attachment of the propeptide and gla domain increases the affinity of KH_2 binding, as contrasted in *A* vs. *B*. As shown in *B* and *C*, multiple carboxylations are accomplished via "tethered processivity," in which the propeptide remains bound throughout the reaction, while the gla domain undergoes intramolecular movement to reposition the glu's for conversion to gla's (γ). During the reaction, the carboxylase is also carboxylated.

range. Most likely, propeptide tethering increases the local concentration of the gla domain with respect to the carboxylase, and the K_m for carboxylation is governed by the propeptide affinity.

Interestingly, covalent attachment of the propeptide to the gla domain results in a second allosteric event, causing an increase in KH_2 affinity (Fig. 1) (11). An effect of gla domain binding on KH_2 activity has been demonstrated in recent studies showing that the peptide FLEEL stimulates epoxidase activity (12). KH_2 activation may require covalent attachment of the propeptide and gla domain either because the contiguous domains alter carboxylase conformation or because the increased affinity for the gla domain leads to increased affinity for KH_2. VKD protein binding and KH_2 activation thus appear to involve two points of contact, one each for the propeptide and gla domain, as shown in Figure 1. The gla domain contact most likely involves binding of the glu's in the active site for carboxylation because a propeptide attached to an all-glu sequence increases KH_2 affinity (13).

Studies with BGP carboxylation support the concept of a glu-binding site. The carboxylase has high affinity for the BGP gla domain (Km = 0.2 μmol/L), at least 850-fold higher than its affinity for the fIX gla domain (14,15). In contrast, the BGP propeptide exhibits poor binding, orders of magnitude less than that observed with other VKD propeptides. The high affinity for the BGP gla domain strongly suggests a binding site for the gla domain of VKD proteins in the carboxylase (Fig. 1). All VKD proteins, then, appear to bind to the carboxylase through low affinity and high affinity sites, with the BGP propeptide and gla domain exhibiting reversed affinity to that of other VKD proteins.

Isolation and Characterization of Recombinant (r)-Carboxylase. Identification of the propeptide as a high affinity ligand for the carboxylase led to its use in purifying the enzyme and cDNA (16–18). The human carboxylase cDNA predicts a unique 758 amino acid protein. An integral membrane protein is predicted; however, the topology of the carboxylase in the membrane is unknown, with different algorithms predicting three or five membrane-spanning sequences in the N-terminus. On the basis of the distribution of predicted glycosylation sites and the large discrepancy between observed (95 kDa) and predicted (78 kDa) molecular weight, the C-terminal half of the carboxylase must be localized in the ER lumen.

Despite the availability of r-carboxylase, the amount of structural information that has been acquired is limited. Given the predicted topologies, a secreted form is probably not obtainable; thus large quantities of protein are still difficult to generate. In addition, the requirement for isolating the carboxylase in a micelle complicates microsequence analysis. To date, mapping has been low in resolution and there have been no reports that biochemically identify functionally relevant amino acids. Binding of a suicide substrate was mapped to the first third of the carboxylase (19). Propeptide binding has been mapped both to amino acids 50–225 by one group (20) and to amino acids 349–500 by another (21). Clearly, a complete understanding of carboxylase structure and function requires the development of techniques that circumvent the problems with mapping residues.

How Are VKD Proteins Multiply Carboxylated? Secreted VKD proteins are normally fully carboxylated, indicating that some mechanism exists to ensure comprehensive carboxylation. Two very different mechanisms could account for full carboxylation. In the first instance, a quality control process could prevent the secretion of uncarboxylated forms. Evidence for cellular regulation exists for rat PT and human PC, in which undercarboxylated forms have been shown to be rapidly degraded (22,23). Rat PT is secreted only in fully carboxylated form. In contrast, undercarboxylated human PT can be secreted, and this specificity is due to sequence differences in the kringle region (24).

In the second instance, full carboxylation could be achieved if the carboxylase is processive, i.e., effecting all modifications as a consequence of a single binding event. Carboxylase processivity has been assessed by analyzing the products of an in vitro reaction between a fIX peptide and the carboxylase (25). A small population of fully carboxylated peptide was detected among a large population of undercarboxylated intermediates, and a probability argument that only processive carboxylation would yield fully carboxylated fIX was used to conclude that the carboxylase is processive. The limited amount of fully carboxylated product is interesting, raising the following questions: Is a cofactor required? Do all VKD proteins demonstrate equal processivity?

Our approach to assess processivity was to develop a direct test in which the carboxylation of a fIX-carboxylase complex was challenged with a distinguishable fIX substrate. We found that carboxylation among individual fIX-carboxylase complexes was nonsynchronous and fully processive, resulting in the comprehensive carboxylation of fIX before the onset of carboxylation of the challenge substrate (Berkner et al., unpublished data). Thus, the VKD protein remains tethered to the carboxylase via the propeptide throughout the reaction, with gla domain intramolecular movement repositioning the glu's for catalysis (Fig. 1). Interestingly, the rate of fIX carboxylation was linear over most of the reaction, which indicates a driving force. The carboxylase must therefore position glu's preferentially for catalysis and hence must distinguish a glu from a gla. Differentiation, e.g., by charge or size, could be accomplished by the proposed glu-binding site.

The concept of a carboxylase site that binds a glu more strongly than a gla is attractive in its ability to explain how the carboxylase modifies the gla domain comprehensively. Processive carboxylation means that the carboxylase does not release a VKD protein until it is fully modified; thus it is able to distinguish "fully" from "partially" modified forms. How this distinction is accomplished is unknown. Full carboxylation

would result in the loss of glu binding (Fig. 1C). This change could effect consequent release of the VKD protein, e.g., due to a conformational change that weakens propeptide binding or due to competition by glu-containing substrates.

Whether the gla domain itself contributes to efficient carboxylation is unknown. Most of the VKD proteins have a highly homologous gla domain, but conservation of structure could be due to a commonality required for biological function rather than carboxylation. Some mutations in the gla domain of r-PC result in the secretion of undercarboxylated protein (26), suggesting that the gla domain is important to carboxylation. However, in another study, a r-PT truncation with the gla domain deleted and the propeptide fused to the serine protease domain was secreted as extensively carboxylated protein (27). Interpretation of these and other studies of secreted VKD proteins is complicated because the ER quality control system may allow only a subset of the intracellular population to be secreted. In an in vitro study, carboxylation of a substrate with the propeptide attached to a random glu-containing sequence resulted in a large proportion of highly carboxylated (i.e., 4 of 5) glu's (15). Thus, the propeptide confers upon a substrate the ability to be multiply modified. However, whether the gla domain contributes to more comprehensive and/or efficient carboxylation remains to be determined.

An in vitro study with BGP indicates that there is some order to the carboxylation of multiple glu's (14). Analysis of the reaction intermediates of BGP, which has only three gla residues per molecule, indicated a preferential order of carboxylation, from the C- to the N-terminus. Given the uniqueness of BGP among the VKD proteins, it will be of interest to determine whether the pattern of carboxylation is the same with other VKD proteins.

Intracellular processing of VKD proteins. How VKD proteins are bound by the carboxylase in the ER, then modified and released, is not well defined (**Fig. 2**). VKD protein-carboxylase association is likely mediated by chaperones, as observed with other multisubunit systems. After carboxylation and release, the VKD protein transits through the Golgi, where propeptide processing occurs. Processing is required for the Ca^{++}-dependent conformational change in the VKD gla domains that results in subsequent membrane binding (28).

The carboxylase has been observed in both the ER and Golgi (29,30), although the significance of this distribution is not known. The carboxylase does not have an identifiable ER retrieval signal; thus it is not known whether carboxylase in the Golgi is subsequently recycled or degraded. One factor that may affect the fate of the carboxylase is its carboxylation. We found that r-carboxylase, expressed in the absence of other VKD proteins, is more rapidly degraded when carboxylated, and that coexpression with another r-VKD protein decreases the rate of degradation. One possibility, then, is that carboxylase carboxylation serves as a signal to regulate the levels of carboxylase, depending upon the availability of vit K or VKD proteins.

Carboxylation of multiple VKD proteins occurs in at least two tissues. Liver synthesizes several VKD proteins (fVII, fIX, fX, PT, PC, PZ, PS and MGP) and osteoblasts express BGP and MGP during healing (31). Tissue distribution of the carboxylase and of several VKD proteins (gas6, MGP, PRGP-1, PRGP-2) has been shown to be broad (4,5,32,33). In addition, recent studies have demonstrated other synthetic sites for the VKD clotting factors (e.g., muscle for PT) (34). Consequently, there may be other tissues that coexpress different VKD proteins.

Carboxylation of multiple VKD proteins in the same tissue suggests the need for regulation to ensure full carboxylation of individual proteins. Given the wide range of VKD propeptide affinities, binding and release of fully carboxylated protein vs. premature release of undercarboxylated protein could differ considerably among the VKD proteins. In vivo studies support the concept that the carboxylase does not process all VKD proteins equally, i.e., analysis of warfarin-treated rat cells expressing PT either alone or with fX suggested that fX outcompetes PT for the carboxylase (35). This interpretation was subsequently supported by in vitro experiments showing a 100-fold difference between fX and PT propeptide affinities (9).

Recent characterization of a hemophiliac highlights the importance of propeptide affinity in the hemostatic process (36). The patient normally exhibited wild-type clotting, but responded to anticoagulation with a selective decrease in fIX activity. An ala to gly mutation at the highly conserved −10 position in the propeptide was identified; a peptide bearing this mutation exhibited a 30-fold decrease in propeptide affinity, but only a twofold decrease in KH_2 binding. To explain the puzzling observation that fIX showed a decrease in activity only during oral anticoagulation, the authors proposed that when KH_2 is limiting, the carboxylation rate slows down and thus the ratio of dissociation rate to catalytic rate (k_{5-7} vs. k_{1-3} in Fig. 2) increases. Thus, fIX would selectively respond to KH_2 depletion due to its lowered affinity.

An additional possibility is that the decreased availability of KH_2 also changes the normal balance between the VKD precursor pool and carboxylase, causing the premature dissociation (k_{5-7}, Fig. 2) of substrates by other VKD proteins with

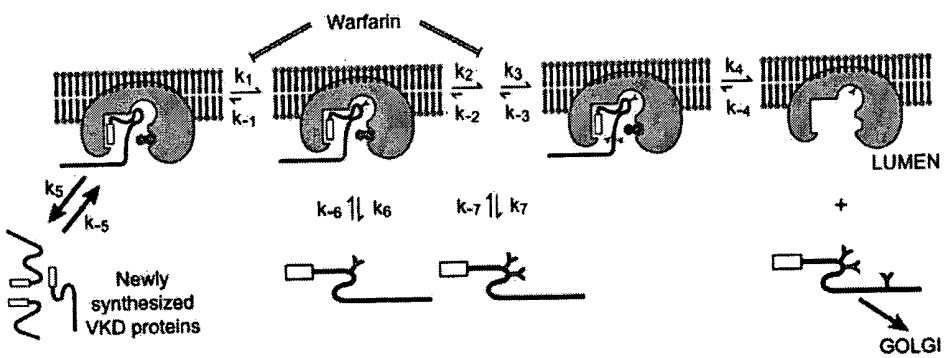


FIGURE 2 Carboxylation and secretion of vitamin K–dependent (VKD) proteins. Normally, VKD proteins undergo comprehensive carboxylation [k_{1-3} in this example for a VKD protein with 3 γ-carboxylated glutamyl (gla) residues], release (k_4) and subsequent propeptide processing in the Golgi. Decarboxylation (i.e., k_{-1} to k_{-3}) is unlikely to be physiologically relevant. Warfarin effects premature release (k_{5-7}) of undercarboxylated forms. This effect could be due to a decrease in k_{1-3} as well as a build-up of VKD precursors that accelerate release. Most VKD proteins also undergo other post-translational modifications, including *N*-glycosylation, *O*-glycosylation, β-aspartyl hydroxylation and proteolytic cleavage at internal sites within the mature protein.

stronger affinities. Interestingly, PT carboxylation is also disrupted only upon anticoagulation, and the PT propeptide has an affinity that is much lower than that of other hepatic VKD propeptides and not very different from that of the fIX mutant (the PT propeptide affinity is 7-fold less than the fIX propeptide (9), and the fIX mutant had a 30-fold lower affinity than native fIX (36)). Under normal conditions, the levels of VKD proteins and carboxylase may be regulated to ensure complete carboxylation. Consequently, efficient carboxylation of VKD proteins with large differences in affinities occurs because a competitive state does not exist. When KH_2 is limiting, decreased VKD protein turnover traps the carboxylase in the complex, and this block results in the accumulation of VKD precursors. Competition between VKD precursors with varying carboxylase affinities can then lead to the displacement of lower affinity substrates such as PT or the fIX mutant.

A disruption in the normal equilibrium between VKD precursors and carboxylase may account for the poor carboxylation that often confounds mammalian expression of r-VKD proteins. Cell lines expressing increased levels of r-VKD proteins become saturated for carboxylation. Cognate cell lines expressing low or high amounts of r-VKD protein have the same amount of carboxylase and vit K; however, there is an increase in the ratio of VKD precursors to carboxylase, which likely compromises the normal secretory process as a result of competition that effects premature VKD protein dissociation. In addition, if k_{cat} in mammalian cells is slow, e.g., due to lack of a vit K reductase that efficiently recycles vit K epoxide, then VKD precursor build-up will compound the problem.

The fact that full carboxylation occurs under normal physiologic conditions is remarkable, and determining what mechanisms effect this process is central to understanding VKD protein carboxylation. For example, is the catalytic rate the same for all VKD proteins or do lower affinity proteins such as PT have a higher k_{cat}? Determining whether there is a control mechanism that regulates the amount of VKD protein available to the carboxylase, and whether other intracellular proteins participate in the process, will also be important in defining the overall process of carboxylation.

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Transgenic pigs as bioreactors: a comparison of gamma-carboxylation of glutamic acid in recombinant human protein C and factor IX by the mammary gland

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Abstract

The mammary gland of transgenic livestock can be used as a bioreactor for producing complex therapeutic proteins. However, the capacity for making a given post-translational modification upon any given polypeptide is uncertain. For example, the efficiency of gamma-carboxylation of glutamic acid in the amino terminal regions of recombinant human protein C (rhPC) and recombinant human Factor IX (rhFIX) is different at similar expression levels. At an expression level of about 200 μg/ml in the milk of transgenic pigs, rhFIX is highly gamma-carboxylated as indicated by pro-coagulant activity and amino acid sequencing. However, only about 20–35% of rhPC has a native, gamma-carboxyglutamic acid-dependent conformation and *anti*-coagulant activity. Thus, this work provides an example of apparent differences in substrate specificity between two homologous proteins to the endogenous carboxylase of porcine mammary epithelium which leads to varying degrees of post-translational modification.
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Keywords: Transgenic pigs; Protein C; Mammary gland

1. Introduction

Mammalian biochemistry uniquely enables the expression of complex recombinant human proteins. A diversity of post-translationally modified polypeptides can be synthesized and secreted from genetically engineered mammalian cells that are cultured in bioreactors [1,2]. However, when the specific rates of synthesis reach about 10 pg/cell/24 h or greater, many rate limitations in post-translational processing begin to be manifested [2]. Most post-translational (PT) modifications are necessary for the native biological activity and also affect the circulation half-life of complex therapeutic proteins. PT-modification of nascent polypeptides is especially necessary for many of the proteins of haemostasis, such as the zymogens human Protein C (hPC) [4] and Factor IX (hFIX) [5]. Removal of the pro-peptide and formation of gamma-carboxyglutamic acid (gla) residues in the amino-terminus of recombinant human Protein C (rhPC) [2,3] and Factor IX (rhFIX) [6–8] are necessary for full *anti*-coagulant and pro-coagulant activities of the activated forms, respectively. Gamma-carboxylation of glutamic acid (gla) is a vitamin K-dependent (VKD) PT-modification, and high levels of carboxylase activity have been found in the human liver and kidney [9]. The VKD family of proteins has a highly conserved propeptide and amino-terminal sequence [10]. In addition, both hPC and hFIX are glycosylated with a complex carbohydrate structure that includes terminal sialic acid moieties on *N*-linked and *O*-linked glycosylation sites [3,5].

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The milk of transgenic rodents such as mice and rabbits and livestock such as pigs, sheep, goats and cows has been engineered to produce a diversity of recombinant proteins (reviewed in Refs. [11,12]). The genetic regulation of mammary epithelium is well conserved among different species and several regulatory sequences from different milk protein genes have been used effectively as transgene promoter elements [12]. For example, the regulatory elements from the mouse whey acidic protein (mWAP) gene have been used to express rhPC [13,14], fibrinogen [15] and human FVIII [16] in the milk of transgenic pigs.

Salient to the investigation of transgenic livestock bioreactor feasibility is the evaluation of the post-translational (PT) processing capacity of the mammary epithelial cell (reviewed in Ref. [11]). It is well known that PT-modification is a diverse but constitutive array of biochemical reactions that can be species-, tissue-, and substrate-specific [1,2]. For that reason, a definitive knowledge of the PT processing capacity for any one type of modification must be evaluated as it occurs for the given polypeptide at a given expression level and within the given cell type. By using transgene constructs having either a 2.5 or a 4.1 kb fragment of the 5′-regulatory sequence from the mWAP gene in combination with either cDNAs or genomic encoding sequences of hPC, we have previously achieved rhPC expression levels of 100 to over 2000 μg/ml in different transgenic swine lineages [13,14]. In this previous work, we have shown that there are rate limitations in gamma-glutamyl carboxylation of rhPC [17,18]. In the present work, a lineage of hFIX transgenic swine that express 100–220 μg/ml of recombinant human FIX is evaluated. A comparison is made of the gla content of rhPC and rhFIX, two homologous VKD proteins, at equivalent expression levels within the milk of transgenic pigs to evaluate the substrate specificity of mammary epithelium for gamma-glutamyl carboxylation.

2. Materials and methods

2.1. Transgene construction and production of transgenic pigs

The structure of the cDNA-based transgene for rhFIX is given Fig. 1. Briefly, a pUC18 cassette vector was constructed containing 2.5 kb of 5′ regulatory sequences of the mWAP gene along with flanking 3′ UTR mWAP sequences (designated pUCWAP5). A Kpn I restriction site was engineered by PCR at the start and stop codons of the 1.4 kb cDNA of hFIX (gift from D. Stafford, University of North Carolina, Department of Biology, Chapel Hill). The modified 1.4 kb cDNA of hFIX was inserted into the Kpn I site of the pUCWAP5 cassette vector to produce the plasmid pUCWAPFIX (Fig. 1). The transgene was excised from the pUCWAPFIX plasmid using *Eco*R I restriction digest and purified using previously reported methods [16]. The structures of the transgenes for producing rhPC are given in [13,14].

A previously reported procedure for producing transgenic pigs by pronuclear injection was used [13,16]. Briefly, about 1–3 pl of 5–10 μg/ml of the transgene construct was introduced into one-cell pig embryos by pronuclear injection. The mWAP-hFIX construct was co-injected with a 7.2 kb genomic mWAP fragment [13]. About 40 microinjected embryos were transferred into recipient gilts that were synchronized as described in previous work [13,16].

2.2. Milk collection and handling

Lactating sows were injected IM with 30–60 IU of oxytocin (Vedco Inc., St. Joseph, MO) to stimulate milk let-down as previously described [13]. Letdown occurred 2–5 min after injection. Pigs were milked by hand during the course of this study. Immediately after collection the milk was diluted 1:1 with 200 mM EDTA, pH 7.0 to solubilize the caseins and then stored frozen. The fat layer was separated by centrifugation at 3000 × *g*, and the skim milk/EDTA fraction was used for all further experiments. In this study, all concentration values reported for milk were obtained from diluted milk/EDTA samples that were multiplied by a factor of 1.9 to account for dilution with EDTA and subsequent removal of milk fat.

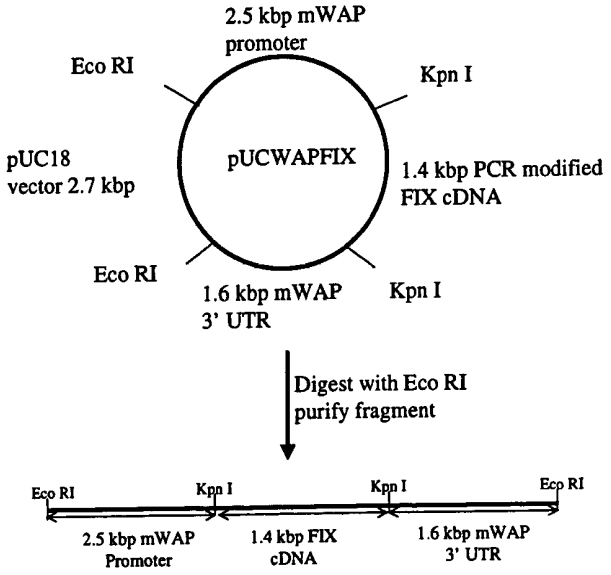


Fig. 1. A 2.5 kbp mWAP-1.4 kbp cDNA rhFIX-1.6kbp mWAP 3′UTR transgene construct.

2.3. Determination of recombinant factor IX in milk

Immulon II microtiter plates (Fisher Scientific, Pittsburgh) were coated overnight with 100 µl/well of 1:1000 rabbit *anti*-human FIX (Dako) in 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6 at 4°C. The wells were washed with TBS-Tween (TBST, 25 mM Tris, 50 mM NaCl, 0.2% Tween 20, pH 7.2), and then blocked for 30 min with TBS/0.1% BSA at room temperature. Samples and hFIX standard (a gift from the American Red Cross) in the TBS-BSA dilution buffer were added in triplicate to the wells (100 µl/well) and incubated at 37°C for 30 min. The wells were then washed and blocked for another 10 min at room temperature. Goat *anti*-human FIX (American Diagnostica, Greenwich, CT), 1:1000 in TBS-BSA, was then incubated in the wells for 30 min at 37°C, followed by *anti*-goat IgG/HRP (Sigma). Bound chromophore was detected with OPD substrate (Abbot, Chicago) at 490 nm using an EL308 Bio-Tek Microplate reader.

2.4. Purification of rhFIX from milk/EDTA

Immunopurification protocols were adapted from previously described methods [18].

All columns and buffers were kept at 4°C. A pool of daily milk/EDTA samples was diluted to OD 280 nm of 5.0 with TBS, pH 7.2, then loaded at 1 cm/min on DEAE FF Sepharose. The column was washed with TBS, pH 7.2, and then eluted with 0.25 M NaCl in TBS. This fraction was diluted 1:1 with 40 mM $MgCl_2$ in TBS to a final concentration of 20 mM $MgCl_2$ and loaded on a metal-dependent immunoaffinity column (Mab 1H5 anchored on Sepharose CL2B, gift from the American Red Cross). The column was washed with TBS containing 20 mM $MgCl_2$, and the product was eluted with 20 mM citrate, 0.15 M NaCl, pH 6.8. The product was dialyzed overnight against 10 mM imidazole, pH 7.2.

2.5. Protein sequencing

Immunopurified rhFIX and hFIX (American Red Cross) were electrophoresed on 16 × 20 cm Bio Rad Protean IIxi 9–18% SDS PAGE, and then blotted onto PVDF membranes (Bio Rad) for sequencing per the manufacturers instructions. The bands on the membranes were excised and then sequenced at the Virginia Tech University Protein Sequencing Laboratory using an Applied BioSystems 477A protein sequencer and 120A analyzer.

2.6. APTT activity assay

The APTT pro-coagulation activity of hFIX samples was used for measuring the specific activity of the immunopurified rhFIX obtained from the milk of transgenic sow 58-1 was adapted from previously reported methods [19]. Standards of normal plasma reference pool (Sigma) and hFIX (American Red Cross Plasma Derivatives Laboratory) were used in the assay. Factor IX depleted plasma was purchased (George King Biomedical; Overland Park, Kansas). Briefly, 0.1 ml of FIX depleted plasma, 0.1 ml of sample in assay buffer, and 0.1 ml of PTT/Protac were added to the assay tubes. The tubes were incubated at 37°C for 3 min, and then 25 mM calcium chloride was added, and the clot formation time was measured using an Elektra 750. Each sample was run in duplicate, with the average of clotting times for three dilutions of each sample used to calculate specific activity. Plots of $\ln\{\text{time}^{-1}\}$ versus $\ln\{[\text{rhFIX}^{-1}]\}$ were used to calculate specific activity. The intercept is related to the K_m and V_{max} for a simplified Michaelis-Menten analysis of the in vitro coagulation process. The slope indicates the relative enzymatic activity.

Table 1
Expression levels of recombinant VKD proteins in transgenic pigs

Transgene construct [promoter]-protein	Single site insertion	Multiple site insertion
[2.5 kb 5'-mWAP] cDNA-hPC[a]	100–500 µg/ml	200–1000 µg/ml
[4.1 kb 5'-mWAP] genomic-hPC[a]	100–300 µg/ml	700–2000 µg/ml
[2.5 kb 5'-mWAP] cDNA hFIX	100–220 µg/ml	Not applicable

[a] Adapted from Van Cott et al. [14].

3. Results

The expression levels of rhPC and rhFIX measured by polyclonal ELISA in the milk of transgenic pigs are shown in Table 1. The levels of rhFIX that were detected in a single transgenic pig having the cDNA transgene ranged from 100 to 220 µg/ml during lactation. This founder female was outbred with a nontransgenic boar. This founder also contained the mWAP gene, which was introduced by pronuclear co-injection along with the rhFIX transgene. About 1 g/l of mWAP protein was also detected in these milk pools. Currently, only the milk of the founder animal has been analyzed. The levels of rhPC ranged from about 100–1000 and 100–2000 µg/ml in milk from pigs having cDNA and genomic rhPC sequences, respectively. Multiple insertion sites were found in the highest expressing rhPC transgenic sows. The lowest levels of rhPC expression of less than about 500 µg/ml were found in sows having a single site of integration [13,14]. Multiple sites of integration were found in one genomic rhPC

Table 2
Amino acid sequencing results of immunopurified rhFIX from the milk of 58-1[a]

Cycle	rhFIX sequencing results		hFIX sequence
	Amino acid	[pmol]	
1	Y	94	Y^1
2	N	74	N^2
3	S	34	S^3
4	G	108	G^4
5	K	62	K^5
6	L	90	L^6
7	E	3*	E^7
8	E	3*	E^8
9	F	64	F^9
10	V	72	V^{10}

[a] The Edman degradation does not recognize gamma-carboxyglutamic acid and therefore the low signal for glutamic acid in cycles 7 and 8 indicate that gamma-carboxyglutamic acid was present at those corresponding amino acid positions.

lineage where a lower gene copy number was found in offspring from the founder animal. The transgenic pigs expressing the lowest level of rhPC (about 100–300 μg/ml) were chosen for the comparison of gamma-carboxylation of rhPC and rhFIX.

Milk samples containing rhFIX were pooled from the first lactation of transgenic sow 58-1. The rhFIX was purified by ion exchange chromatography followed by metal-dependent immunoaffinity chromatography (MAb 1H5, see Ref. [19]). The yields from the anion exchange and immunoaffinity steps were quantitative, with less than 5% of the rhFIX detected in the flow-through chromatographic fractions by polyclonal ELISA. Using this two-step chromatographic procedure, we were able to purify the rhFIX to about 98% purity, as judged by the silver stained SDS PAGE. The *N*-terminal amino acid sequence of immunopurified rhFIX obtained from western transfer of a major band occurring at about 65–75 kDa under nonreducing conditions is given in Table 2. The *N*-terminal amino acid sequence of the purified rhFIX corresponded to the *N*-terminal sequence of human FIX [5], and matched the sequence of purified human FIX standard obtained from the American Red Cross. The lack of a strong glutamic acid signal in cycles 7 and 8 of the purified rhFIX sample is noted. Glutamic acid residues that are gamma-carboxylated can not be detected by the amino acid sequencer.

The biological activity of the purified rhFIX was measured by a one-stage APTT clotting assay. The immunopurified rhFIX had a specific activity of 337 U/mg, the immunopurified hFIX from plasma had a specific activity of 230 U/mg, with the normal plasma reference pool activity defined as 250 U/mg (Table 3). Plots of natural log [time^1] versus natural Log [rhFIX^{-1}] resulted in similar intercepts for NPRP, purified hFIX, and the immunopurified rhFIX. The intercept is related to the K_m and V_{max} for a simplified Michaelis-Menten analysis of the in vitro coagulation process. The slope indicates the relative enzymatic activity.

4. Discussion

The production of rhPC in the milk of transgenic pigs at levels of about 100–500 μg/ml was typical for lineages having a 2.5 kb-5′-WAP regulatory sequence, a cDNA encoding sequence and a single site of integration [13,14]. In general, transgenic sows with these constructs having multiple sites of integration gave expression levels of 100–500 μg/ml per site of integration. Thus, the 100–220 μg/ml expression level of the 2.5 kb-5′WAP:cDNA-hFIX transgene was not unexpected as the mWAP is transcriptionally active in many of the transgenic pigs evaluated. Since the pig has milk let-down about every hour, this expression level translates to about a 1–10 pg/cell/day synthesis rate assuming no intracellular accumulation [17]. The absence of the previously reported cryptic splice acceptor site occurring in flanking 3′-UTR of hFIX sequences may have been an enabling element for the much higher expression levels than achieved in transgenic mice and sheep [20,21]. The expression of only 25 ng/ml or less was previously reported in sheep using transgenes with 3′-UTR sequences from hFIX [20]. It is possible that genomic DNA sequences of hFIX would have given expression levels of 1000 μg/ml or higher in the milk of swine for lineages with single insertion sites. However, our previous knowledge of the rate limitations in post-

Table 3
APTT coagulation assay of immunopurified rhFIX from the milk of 58-1[a]

Sample	Slope	Slope ratio	Equation	% Activity	Specific activity
NPRP	0.094	1.0	$y = 0.094x - 3.7$	100	250 U/mg
hFIX (ARC)	0.086	0.92	$y = 0.086x - 3.6$	92	230 U/mg
58-1 rhFIX	0.127	1.35	$y = 0.127x - 3.4$	135	337 U/mg

[a] A one stage clotting assay was performed according to the protocol given by the American Red Cross Plasma Derivatives Laboratory (procedure for Factor IX coagulation assay, March 1992).

Table 4
Amino acid sequences of PC and FIX propeptides and Gla domain

	−14	−13	−12	−11	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1						
PC	S	S	E	R	A	H	Q	V	L	R	I	R	K	R						
FIX	D	H	E	N	A	N	K	I	L	N	R	P	K	R						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PC	A	N	S	F	L	*E*	*E*	L	R	H	S	S	L	*E*	R	*E*	C	I	*E*	*E*
FIX	Y	N	S	G	K	L	*E*	*E*	F	V	Q	G	N	L	*E*	R	*E*	C	K	*E*

translational modification for performing gamma-carboxylation in rhPC [17,18] suggested that a cDNA based construct using the 2.5 kb-5′-WAP regulatory sequence would more likely yield a higher percentage of properly formed gla-residues at expression levels of less than 500 μg/ml. This proved to be correct with gamma-carboxylation occuring at high efficiency on the rhFIX recovered from pig milk.

The recovery of 85% or more of rhPC from the milk of transgenic sows producing about 100–1000 μg/ml has been previously reported [18]. In that study, we showed that the percentage of properly gamma-carboxylated rhPC was inversely proportional to expression level. For example, at expression levels between 100–300 μg/ml a minimum of 35% of the rhPC was immuno-purified by an *anti*-hPC monoclonal antibody which was sensitive to the calcium-dependent conformation of the gla domain. The relative amounts of rhPC populations having a calcium-dependent conformation that were recovered by immunoaffinity were consistent with the relative amounts of properly gamma-carboxylated rhPC. The recovered populations gave about 70–160% of theoretical specific anticoagulant activity in an APTT assay and were properly carboxylated at amino acid positions 6 and 7 as determined by amino acid sequencing [17–19].

Both rhPC and rhFIX have been produced in several different mammalian cell lines such as baby hamster kidney cells, human kidney 293 cells, Chinese hamster ovary cells and adenovirus-tranformed Syrian hamster cells [2,6,7]. Each of these cell lines exhibited limitations in gamma-carboxylation at a rate of synthesis of about 1–10 pg/cell/24 h. The mammary epithelial cells of the transgenic pig show similar limitations in PT-formation of gla residues in the light chain of rhPC at a similar rate of synthesis [17,18]. In contrast, yields from rhFIX purification (which used a conformational dependent monoclonal antibody that recognizes fully gamma-carboxylated FIX; see Ref. [19]), from amino acid sequencing of the *N*-terminus of purified rhFIX, and from APTT coagulation assays indicated that rhFIX is fully gamma-carboxylated. Furthermore, the removal of propeptide sequences in rhPC and rhFIX was found in those populations that were highly active. Table 4 shows that rhFIX and rhPC have over 80% conservation of primary structure in the propeptide and amino-terminal gla-domain. However, differences in amino acid side chain charge and hydrophobicity are noted at positions −4 and −3 in the FIX propeptide, and the gla domain of FIX is shifted with respect to hPC by the insertion of a glycine at +4. Thus, it is possible that small differences in primary structure of the nascent rhPC and rhFIX polypeptides can result in unpredictable PT-processing efficiencies for a given PT-modification. The higher percentage of carboxylated rhFIX than rhPC in the milk of transgenic mice [21] and pigs suggests that rhFIX may be inherently more amenable to carboxylation than rhPC. This may be a result of the differences in substrate specificity for the putative carboxylase that is endogenous to the mammary epithelium in these two species.

This work suggests that a trial production of each recombinant protein in the milk of the transgenic livestock of interest may be necessary in order to deduce the capacity for PT-modification with any degree of certainty for a given complex protein. For those polypeptides where PT-modification is rate limiting, enhanced PT-processing may be performed through transgenesis. For example, the addition of the *endo*-protease furin under the regulatory control of the mWAP gene was used to enhance propeptide removal from rhPC expressed in the milk of mice [22].

While this work establishes the rate limitation of gla formation with expression level, we are presently studying the relationship between sialic acid content and expression level. Initial results indicate that low sialic acid content and gla content are correlated in the same sub-fractions of rhPC that were isolated by free-flow isoelectric focusing (unpublished observations).

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